Cell Proliferation

Cell Prolif. 2008, **41**, 348–364 doi: 10.1111/j.1365-2184.2008.00515

Collagens, stromal cell-derived factor-1α and basic fibroblast growth factor increase cancer cell invasiveness in a hyaluronan hydrogel

L. David*, V. Dulong†, B. Coquerel*, D. Le Cerf†, L. Cazin*, M. Lamacz* and J.-P. Vannier*

**Groupe de Recherche sur le Micro-Environnement et le Renouvellement Cellulaire Intégré (M.E.R.C.I., UPRES EA 3829), Faculté de Médecine Pharmacie, Université de ROUEN, Rouen, France, and †Laboratoire PBM – UMR 6522, CNRS, Université de ROUEN, Mont Saint-Aignan, France*

Received 22 *August* 2007*; revision accepted* 14 *September* 2007

Abstract. *Objective*: Beyond to control of cell migration, differentiation and proliferation, the extracellular matrix (ECM) also contributes to invasiveness of human cancers. As the roles of hyaluronan (HA) and collagens in this process are still controversial, we have investigated their involvement in cancer pathogenesis. *Materials and methods*: With this aim in view, we developed a three-dimensional matrix, as reticulate HA hydrogel alone or coated with different collagens, in which cells could invade and grow. *Results*: We show that cancer cells, which were non-invasive in a single HA hydrogel, acquired this capacity in the concomitant presence of type I or III collagens. Both types of ECM compound, HA and collagens, possess the capacity to stimulate production of metalloprotease-2, recognized otherwise as a factor for poor cancer prognosis. HA-provoked cellular invasiveness resulted from CD44-mediated increase in cytosolic [Ca^{2+}] and its subsequent hydrolysis due to ADAM (a disintegrin and metalloprotease) proteolytic activity. Interestingly, this mechanism seemed to be absent in non-invasive cancer cell lines. *Conclusion*: Furthermore, using basic fibroblast growth factor and stromal cell-derived factor-1α, we also show that this three-dimensional reticulate matrix may be considered as a valuable model to study chemokinetic and chemotactic potentials of factors present in tumour stroma.

INTRODUCTION

Behaviour of normal and tumour cells is directly conditioned by composition of the extracellular matrix (ECM) in which hyaluronan (HA) and collagens are the principal constituents. It is generally accepted that HA, a glycosaminoglycan composed of a repeating disaccharide of glucuronic acid and N-acetylglucosamine (β1,4-GlcUA-β1,3-GlcNAc-)$_n$, is implicated in tumour progression (Stern 2005). High content of intracellular HA and its accumulation in

Correspondence: Laurent David, Groupe M.E.R.C.I., Faculté de Médecine-Pharmacie, 22, Boulevard Gambetta, 76 183 Rouen, France. Tel.: (33) 2 35 14 83 50; Fax: (33) 2 35 14 83 40; E-mail: laurent.david@etu.univ-rouen.fr

the ECM create a microenvironment favourable for migration, proliferation and invasiveness of malignant cells (Delpech *et al*. 1997a; Toole *et al*. 2002). Thus, invasive capability depends on the interaction with ECM and is promoted by HA production as shown in colon carcinoma (Kim *et al*. 2004), breast adenocarcinoma (Auvinen *et al*. 2000) and gastric cancers (Vizoso *et al*. 2004). Stromal accumulation of HA correlates with poor prognosis in breast and gastric cancers (Ropponen *et al*. 1998; Auvinen *et al*. 2000). Activities of cancer cells are controlled by transductional mechanisms involving HA membrane receptors such as RHAMM or CD44, CD44, which is both a ubiquitous cell surface adhesion molecule and the main receptor for HA, is implicated in cell-to-cell and cell–ECM interactions and cell migration (Assmann *et al*. 2001; Knudson *et al*. 2002; Ponta *et al*. 2003; Kim *et al*. 2004). However, little is known concerning regulation mechanisms of CD44-HA in tumour cells (Kincade *et al*. 1997).

The invasive potential of malignant cells also reflects their ability to secrete different types of matrix metalloproteinases (MMP) that, by inducing proteolytic collagen hydrolysis, are responsible for reorganization of the ECM. Because MMP-2 and MMP-9 are known to lead to cell intra- and extravasation, after degradation of types IV and V collagens in vessel basal membranes and secretion of gelatinases is considered as unfavourable for prognosis in cancer development (Foda & Zucker 2001; Lakka *et al*. 2004). MMPs can mediate tumour invasion through several mechanisms including: (i) constitutive production of enzymes by tumour cells, (ii) induction of MMP production in neighbouring stromal cells and (iii) interactions between tumour and stromal cells, to induce MMP production by one or both cell types (Klein *et al*. 2004; Turpeenniemi-Hujanen 2005).

Inability to assess cells' malignant potential *in vitro* can be attributed to lack of a three-dimensional ECM model in which to observe them, as migration of cancer cells triggered by chemotactic and chemokinetic agents requires a highly coordinated sequence of attachment and detachment events provided by components of a three-dimensional matrix (Maaser *et al*. 1999; Ferris & Wang 2003; Eisenhardt *et al*. 2005). One of the most efficient stimulators of cell migration is largely described as the stromal cell-derived factor-1α (SDF-1α)–CXCR4 axis (Kucia *et al*. 2004). Aberrant expression of CXCR4, a highly selective receptor of SDF-1α, confers to several cancer cell types the ability of migration towards an SDF-1α gradient-determining metastasis tissue preference. Invasive tumour cells derived from non-small cell lung cancer have been shown to express this receptor in high numbers (Su *et al*. 2005) and the same role has also been attributed to basic fibroblast growth factor (bFGF), also known as a powerful angiogenic (Presta *et al*. 2005) and growth factor (Chandler *et al*. 1999). Several studies have revealed that bFGF receptors are expressed in highly aggressive cancer cell lines and more particularly in the early phase of cancer installation (Gontero *et al*. 2004).

Recently, our team has developed a three-dimensional hydrogel composed of HA, in which invasive capacity of several cancer cell lines was evaluated (David *et al*. 2004). We have shown that among 12 cancer cell lines, only four were able to colonize this hydrated matrix reticulated exclusively by HA. The other cell types, despite their invasive character observed *in vivo*, exhibited only very weak capacities to do this and two of them were practically unable to survive in the matrix. In the present study, we report data obtained with cell lines, having either pronounced or very weak capacity of invasiveness. Cells were cultured in three-dimensional HA matrix coated with type I, III, IV or V collagens. We observed significant effects of all collagens on increase in invasiveness of weak capacity cells and only slight effects of types I and III collagens on high invasive potential. These new three-dimensional matrix models were also employed to define whether SDF-1α and bFGF act as chemotactic and/or chemokinetic factors.

MATERIALS AND METHODS

Synthesis of hyaluronan and collagen/hyaluronan hydrogels

The HA hydrogels used consisted of a long chain of HA cross-linked with adipic dihydrazide (ADH) (Sigma, Saint Quentin Fallavier, France) as the cross-linking agent and 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDCI) (Sigma) as reagent. All hydrogels were prepared from high molecular weight ($> 1 \times 10^6$ Da) HA (Sigma), similar to the procedure described previously by Prestwich *et al*. (1998). Briefly, ratios of ADH to HA and HA to EDCI were adjusted to obtain hydrogels optimized for cell adhesion and culture. The best results were obtained with ratios of ADH to HA equal to 10 : 1 and ratios of HA to EDCI equal to 1 : 1. HA and hydrazide cross-linker (ADH) were dissolved in MilliQ water and pH was adjusted to 4 by adding 0.1 N HCl. The carbodiimide reagent (EDCI) was dissolved in MilliQ water was added to the mixture, and allowed to gel for 2 h with gentle agitation. HA hydrogels were equilibrated in 0.1 N NaCl for 2 days, then in a mixture water/ethanol (3/1, v/v) for 2 days, and in MilliQ water for 2 days to remove ADH. HA hydrogels were lyophilized, then stored. Before use for cell culture, squares of the HA hydrogels were cut (to approximately $5 \times 5 \times 1.5$ mm) then sterilized (100 °C for 30 min) and were rehydrated with culture medium; they swelled roughly to 100-fold in mass. After rehydratation, properties of the HA hydrogels were similar to the properties before lyophilization.

For preparation of the collagen/HA hydrogel, all hydrogels were prepared similar to the procedure described previously by Tun *et al*. and coating of collagen was performed by immersing HA hydrogels in 0.06% collagen (type I, III, IV or V) for more than 3 h (Tun *et al*. 2002). After collagen coating, each hydrogel was washed with phosphate-buffered saline (PBS) (Eurobio, Les Ulis, France) three times, and PBS inside the hydrogels was removed by dialysation with RPMI-1640 medium (Bio-Whittaker, Verviers, Belgium). Amounts of coated collagen were estimated by the Bradford method after mechanical degradation of the hydrogel.

Cancer-derived cell lines culture conditions

Cell line CB191 was established in our laboratory from gliomas. Cell line SA87 was derived from a brain metastasis developed in a patient with a gastric adenocarcinoma (Maunoury 1977) and lines SA87M1 and SA87M2 were derived from hepatic metastases obtained from nude mice in our laboratory. All cultures were made in RPMI-1640 medium with 2 mM glutamine (Bio-Whittaker) and 10% foetal calf serum (Eurobio) and were propagated in a humidified atmosphere under 5% CO_2 in air at 37 °C. In all cases, cell proliferation was carried out in 75 cm^2 Falcon flasks. Culture medium was changed twice weekly, and cells were trypsinized at confluence with 0.05% trypsin, 0.02% EDTA solution in Ca^{2+}- and Mg^{2+}-free balanced salt solution (Bio-Whittaker).

CD44 localization

Cells were seeded on 1.5-cm^2 glass slides, dried and stored. For staining, they were rehydrated in PBS, then were incubated with anti-CD44H antibody (CD44H, Serotec, France; dilution 1/50), for 1 h at 37 °C in a humidified atmosphere. Control cells were incubated with antiglial fibrillary acidic protein (GFAP) monoclonal antibody (Serotec). After three washings with PBS, cells were incubated with biotinylated antimouse IgG (H + L) (Vectastain®, Vector, Burlingame, CA, USA) at a dilution of 1/200 for 15 min at 37 °C and were washed again with PBS. Cells were then incubated with AP-streptavidin at a dilution of 1/500, for 5 min at 37 °C. Enzyme staining was performed with naphthol-fast-red levamisole. To determine the proportion of cells

expressing CD44, six random fields were analysed in each slide. The total number of cells was determined in each field as well as the number of cells expressing CD44.

Hyaluronan-binding sites

Cancer cells (~6×10^4 cells) were seeded on 1.5-cm^2 glass slides and then, 48 h later, were treated with *Streptomyces* HAse (Calbiochem, France Biochem, Meudon, France, 10 TRU/mL) for 30 min at 37 °C in humid atmosphere, to degrade HA produced by the cells. Control cells were treated for 30 min at 37 °C with *Streptomyces* HAse at a concentration of 10 TRU/mL, to degrade HA before incubation with the hyaluronectin (HN) probe. Cells were washed gently three times with RPMI, and HA fixation was determined by incubating the cells (30 min at 37 °C in humid atmosphere with 5% CO_2) with exogenous HA (10 μg/mL) diluted in RPMI. Cells were fixed with acaetic alcohol, and HA was localized using biotinylated HN as a probe. HN is a glycoprotein, which binds specifically to HA and whose peptide sequence was similar to the sequence of the HA-binding moiety of the proteoglycan PG-M/versican (Delpech *et al*. 1997). HN was purified from sheep brain as previously described (Delpech *et al*. 1995). Biotinylation of HN was carried out using an Amersham kit (Saclay, France). Cells were incubated with the HN probe (5 μg/mL) for 30 min at 37 °C. They were then washed three times with PBS and incubated with AP (alkaline phosphatase)-streptavidin (AP, Boehringer, Meylan, France) at a dilution of 1/5000 for 5 min at 37 °C. Staining was carried out using naphthol-fast-red levamisole at a concentration of 6 mg/mL in Tris buffer. Cells were also stained with haematoxylin (Sigma) to visualize cell nuclei. The proportion of HA-binding site-positive cells was determined as for CD44.

Invasiveness of cell lines in hydrogel

Cancer cells were trypsinized, centrifuged at 300 ***g*** for 10 min and the pellet was re-suspended in culture medium to disperse cells for counting. The 10^6 cells in 2 mL RPMI 10% foetal calf serum (FCS) were seeded in a well containing either a HA hydrogel (control) or a collagen-coated HA hydrogel. Cells were allowed to migrate and colonize the hydrogel for 24 h in humidified atmosphere under 5% CO_2 in air at 37 °C. Then, the hydrogel was transposed in a new well with fresh culture medium and incubated in humidified atmosphere under 5% CO_2 in air at 37 °C for 5 days. Experiments were performed in duplicate. Hydrogels were examined using an inverted microscope. Colony counting was performed using a computerized morphometric system and Cyberview 3.0 program (Cervus International, Courtaboeuf, France) plus CCD camera. The program was calibrated for each magnification. Measurements were performed by tracing diameters of colonies on digitalized images, colonies of more than 150-μm diameter being scored. Thus, invasiveness of cancer cell lines was estimated by ability of cells to invade through reconstituted HA matrix.

Cell adhesion assay

Multiwell plates were incubated with 100 μL of collagen solution (0.06% in PBS) for 24 h at 4 °C. Negative control wells were incubated with 2% bovine serum albumin and positive control wells were incubated with PBS. Wells were washed three times with PBS and no specific binding was blocked by 2% bovine serum albumin for 30 min before addition of cells at 10^5/well in RPMI. Cells were allowed to adhere for 2 h at 37 °C. Non-adherent cells were removed by washing three times with RPMI. The remaining adherent cells were fixed in acetic alcohol for 15 min and stained with 0.1% crystal violet. The stain was eluted from the cells with 10% acetic acid and the absorbance read at 590 nm. Data were recorded as the number of adhesive cells/number of input cells × 100.

Analysis of MMPs by zymography

After colony counting, the hydrogels were washed three times in PBS in order to eliminate FCS and incubated in serum-free RPMI for 48 h. Gelatinolytic activities in these cell-conditioned media were analysed under non-reducing conditions using substrate (gelatin) sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (zymography) as described (Janowska-Wieczorek *et al*. 1999). Samples (20 μg protein/sample) mixed with loading buffer, were applied to a 10% polyacrylamide gel copolymerized with 1 mg/mL gelatin (Sigma) and electrophoresed at constant voltage. The gels were washed in 2.5% Triton X-100 (Sigma) for 1 h to remove SDS and incubated in zymography buffer (50 mM Tris/HCl, 5 mM $CaCl_2$ pH 7.6) for 24 h at 37 °C. Then, the gels were washed with unionized water and stained with 0.05% Coomassie brilliant blue G (Sigma) in 25% ethanol, 10% acetic acid water, and destained in 5% methanol, 7% acetic acid water. The gelatinase activities appeared as clear bands against a blue background. A serum 10% sample was used as positive control.

Measurement by microfluorimetry of cytosolic calcium variations in the presence of HA

For microfluorimetric studies, tumour cells were cultured on 25 mm diameter coverslips. The cells were incubated in the dark with 5 μM indo-1/AM (Molecular probes, Leiden, the Netherlands) in RPMI supplemented with 15 mM HEPES (Sigma) medium at 37 °C for 30 min. At the end of the incubation period, the cells were washed twice with 2 mL of fresh medium. The intracellular calcium concentration $[Ca^{2+}]_i$ was monitored by dual-emission microfluorimeter system constructed from a Nikon Eclipse TE300 inverted microscope (Nikon, Champigny sur Marne, France), as previously described (Galas *et al*. 2000). The fluorescence emission of indo-1, induced by excitation at 355 nm, was recorded at two wavelengths (405 and 480 nm) by separate photometers (Nikon). The 405/480 ratio, was obtained by PC program developed by Notocord Systems (Croissy sur Seine, France). All three signals, corresponding to the absorbance at 405 and 480 nm, and the 405/480 ratio, were continuously recorded by PC-assisted system developed by Notocord Systems. The actual values of $[Ca^{2+}]_i$ were calculated from the following formula established by Jackson *et al*. (1987) $[Ca^{2+}]_i = Kd \times \beta(R - R_{min})/(R_{max} - R)$ where R is the 405/480 nm ratio, R_{min} represents the minimum fluorescence ratio obtained after the incubation of cells with 10 mM EGTA and 10 μM ionomycin for 3 h, R_{max} represents the minimum fluorescence ratio obtained after the incubation of cells with 10 mM $CaCl_2$ and 10 μM ionomycin for 3 h, and β is the ratio of fluorescence yield from the $Ca^{2+}_{min}/Ca^{2+}_{max}$ indicator at 480 nm. The average values of R_{min}, R_{max} and β were 0.14, 2.02 and 1.59, respectively. Kd = 250 nM is the dissociation constant for indo-1/AM (Jackson *et al*. 1987). HA was injected in the vicinity of the cells by a pressure-ejected system. Student's *t*-test was used for the statistical evaluation of the area under curves, calculated using Excel program.

Effect of TAPI on invasiveness of cell lines

Cells were prepared as described previously. TAPI [an ADAM (*a disintegrin and metalloprotease*) *inhibitor*, Calbiochem, France] was added to the culture medium (200 μM). Then, 10^6 cells were seeded in the liquid phase of a well containing a piece of the HA hydrogel. As described above, cells were allowed to migrate and colonize the HA hydrogel. Then, the HA hydrogel was transposed in a new well containing RPMI (10% FCS) and colonies were scored after 5 days of culture.

Effect of SDF-1α and bFGF on invasiveness of cell lines

Cells were prepared as described previously in order to study two types of cell movements, the chemotaxis that is defined as directional cell movement towards concentration gradients of solubilized attractants, and the chemokinesis that is defined as random cell movement in the

absence of chemoattractant gradients (Liu & Klominek 2004). Then, 10^6 cells were seeded in the liquid phase of a well containing a HA hydrogel and the cytokine, SDF-1α (100 ng/mL) or bFGF (25 ng/mL), were directly added to the supernatant or in both supernatant and HA hydrogels. For the latter and before culture, the hydrogels were pre-incubated with the cytokines for 24 h. As described above, cells were allowed to migrate and colonize the HA hydrogel. Then, the HA hydrogel was transposed in a new well with fresh culture medium and colonies were scored after 5 days of culture.

Statistical analysis

To determine the significance of the observed differences in cell culture in the HA hydrogels, the Mann–Whitney *U*-test (two groups) or the Kruskal–Wallis test (from three groups) were employed, assuming unequal variance and two-tailed distribution. All results were reported as mean ± standard error mean. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Invasiveness of cell lines in collagen/hyaluronan hydrogel

In order to correlate invasive potential of cancer cells with a type of collagen, we compared invasiveness of four cell lines (SA87, SA87M1, SA87M2 and CB191) that were cultured in reticulate HA hydrogel alone or coated with type I, III, IV or V collagens and gelatin.

Gradual invasion by malignant cells led to formation of clusters (< 150 μm diameter) and colonies (> 150 μm diameter). Results obtained are illustrated by the histograms shown in Fig. 1. It can be observed that number and shape of colonies depended on the type of the cell line (Fig. 2 and Table 1). Less invasive cells formed only few round colonies, whereas more invasive cells formed numerous, large and irregular colonies. Previous experiments have shown that a 5-day period was appropriate to evaluate invasiveness of the studied cells. Mean number of colonies detected in the HA hydrogel after 5 days of culture ranged from 19 to 258 according to composition of the hydrogel (Fig. 1a). We first estimated quantity of collagen adsorbed on the HA hydrogels in order to determine whether differences could simply be due to concentration of different collagens within the hydrogel. Then, we measured the same concentration for the four tested collagens (between 143.34 ± 13.82 and 168.47 ± 13.85 μg per hydrogel). Therefore, differences in invasiveness were not due to collagen concentration in the hydrogel, but to the collagen type.

Table 1. Colony morphometry

	Colony size (μm)	Colony perimeter (μm)
SA87	248.79 ± 12.38	503.73 ± 55.33
SA87M1	203.83 ± 37.89	408.02 ± 52.66
SA87M2	229.12 ± 57.00	466.59 ± 41.84
CB191	127.14 ± 52.08	331.94 ± 37.72

The hydrogels were examined using an inverted microscope. Colony counting was performed by a computerized morphometric system. The program was calibrated for each magnification. The measurements were performed by tracing the diameter and the perimeter of colonies on digitalized images.

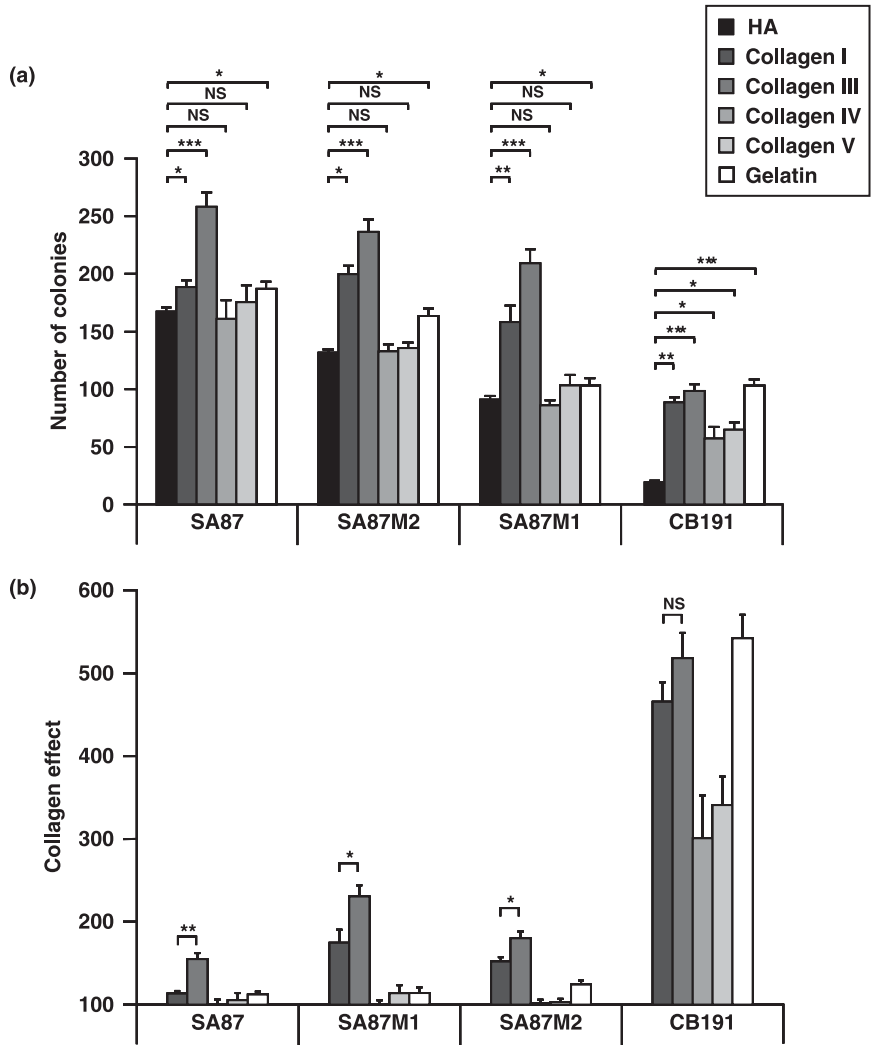

Figure 1. Invasiveness of cancer cell lines in single HA hydrogels and in hydrogel coated with different types of collagen. (a) Number of cancer cell colonies of more than 150 μm diameter was determined for each hydrogel at day 5 using an inverted microscope. Results represent number of colonies per hydrogel as the mean ± SEM of values obtained from five independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$). (b) Results represented according to type of collagen and normalized as the number of colonies in the hydrogel coated with collagen or gelatin to number of colonies in pure HA hydrogel (corresponding to 100%).

As can be observed from Fig. 1b, types I and III collagens are potent activators of cancer cell invasiveness. These effects were particularly powerful in CB191 cells where stimulation level corresponded to ≈ 500% of increase in the number of colonies ($P < 0.0001$). Except for with CB191 cells, the effect of type III collagen was stronger than that induced by type I collagen (on average, +45%, $P < 0.05$). Types IV and V collagens, except for marked stimulation of CB191 cells ($P < 0.05$), were devoid of capacity to increase cell invasion. On the other hand, CB191 cells also were stimulated significantly by the matrix containing gelatin ($P < 0.0001$) and

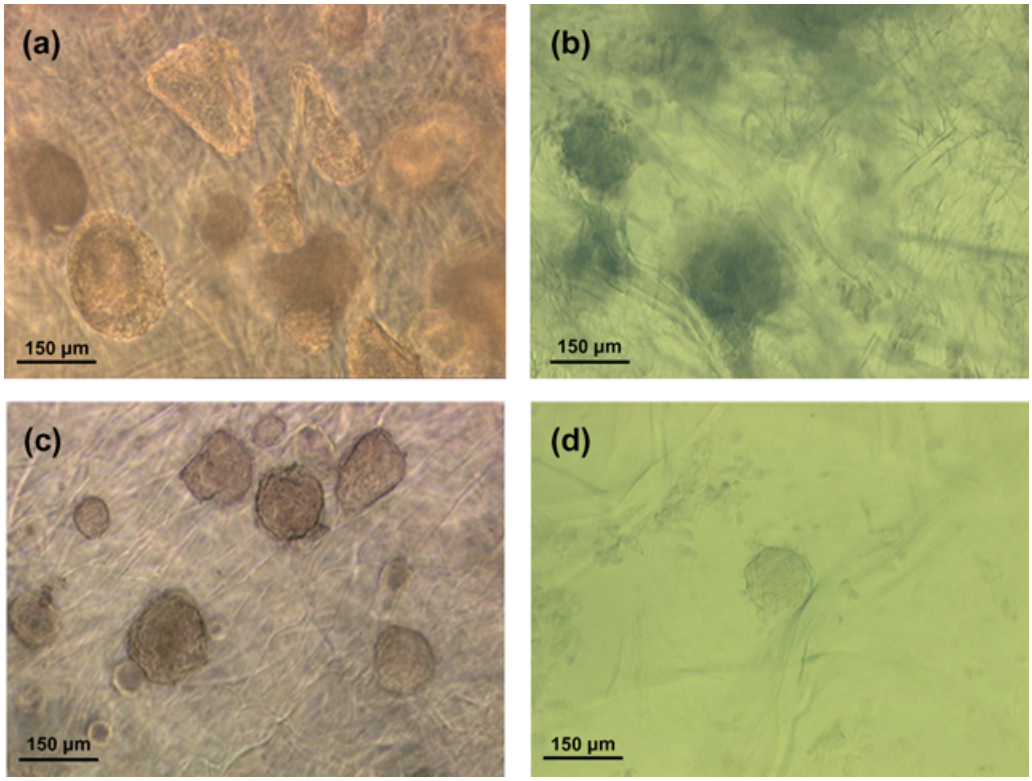

Figure 2. Cancer cell lines in HA hydrogels. General aspect of gastric adenocarcinoma SA87 (a), SA87M1 (b), SA87M2 (c) and glioblastome CB191 (d) cell colonies in the HA hydrogels, shown at the 5th day after migration. Magnification ×100. A representative experiment is shown for each cell type. Bars = 150 µm.

the number of colonies formed by SA87, SA87M1 and SA87M2 cells was stimulated slightly ($P < 0.0278$, $P < 0.05$ and $P < 0.0155$).

Cancer cell adhesion to collagens-coated plates

We tested the hypothesis that collagen type could influence binding ability of cancer cells. Multiwell plates were coated with collagen (type I, III, IV or V) and the number of cells adhering to these surfaces was determined. For collagen type studied, cell adhesion to collagen-coated wells was higher or equal than that of control wells, without collagen. However, there were no significant differences in adhesion found between the four tested collagens (Table 2); thus, results for different collagens were not due to the initial adherence of the cells but to the continuous signalling exerted during the later growth.

Zymography studies; MMP-2 and MMP-9 in cell supernatants

SA87 and CB191 cells were cultured in normal medium, on uncoated plates, in the presence of HA hydrogel alone (HA) or hydrogel coated with one type of collagen (I, III, IV or V) or gelatin (G) for 5 days. Control cells and hydrogels were washed three times in PBS in order to eliminate FCS, and control cells and the hydrogels were then incubated in serum-free RPMI for 48 h. In order to determine whether secretion of MMPs may be involved in observed invasiveness, supernatants of cultured cells were analysed using the zymography approach. As summarized in

Table 2. Adhesion of cancer cells to collagen-coated surface

	Control (% of cells)	Type I collagen (% of cells)	Type III collagen (% of cells)	Type IV collagen (% of cells)	Type V collagen (% of cells)
SA87	58.58 ± 0.83	59.74 ± 1.54	61.86 ± 1.74	57.55 ± 1.27	58.02 ± 0.84
SA87M1	59.90 ± 0.90	60.53 ± 1.06	60.37 ± 0.81	58.97 ± 1.05	59.58 ± 1.12
SA87M2	59.08 ± 0.92	58.46 ± 0.74	59.68 ± 1.45	57.78 ± 0.86	58.73 ± 0.79
CB191	61.31 ± 1.20	60.62 ± 1.08	61.96 ± 0.66	66.60 ± 1.17	64.80 ± 1.00

Cells were seeded on collagen-coated wells or BSA-coated control wells and allowed to adhere for 2 h. At the end of incubation, adherent cells were stained and the absorbance was read at 590 nm. Results are expressed as mean ± SEM of triplicate determinations. Data were recorded as the number of adhesive cells/number of input cells × 100.

Table 3. CD44 and HA-binding sites on cancer cell lines

		HA-binding sites	
	CD44 (% of cells)	Control (% of cells)	TAPI 200 μM (% of cells)
SA87	95	0	48**
SA87M1	92	0	41*
SA87M2	90	0	37*
CB191	100	95	95

Cells were analysed for the presence of CD44 and HA-binding sites. Individual cells incubated on glass slides were screened for CD44 with anti-CD44 monoclonal antibody and for HA-binding sites with HN–PA (after digestion with HAse to remove endogenous HA) as described in 'Materials and Methods'. The percentage of cells on the slide that have CD44 or HA-binding sites is given (the experiments were performed twice). TAPI versus control $*P < 0.05$ and $**P < 0.001$.

Fig. 3, both cell lines used in this experiment were able to secrete small amounts of MMP-2 and MMP-9 in the absence of any ECM element (i.e. on uncoated plates). Then, they were able to respond to ECM elements (type I or III collagen, gelatin) by an increase of MMP-2 secretion with no significant modification in MMP-9 level. Here, it should be noted that if type I collagen seemed to provoke the strongest effect on MMP-2 secretion, types IV and V collagens were completely devoid of any stimulatory action (data not shown).

CD44 expression and cytosolic calcium mobilization by HA

As migration of malignant cells was shown to be dependent on the presence of CD44, the main HA receptor (Naor *et al*. 2002; Toole *et al*. 2002; Marhaba & Zoller 2004), we investigated expression of this receptor in four cell lines. Immunohistochemical analysis showed that practically all cells were CD44-positive (90–100%; Table 3). Nevertheless, this did not mean that these lines could really bind HA, as some CD44 isoforms are known to be devoid of binding capacity. Indeed, CD44 exists in three states: active, inactive and inducible (Nagano & Saya 2004). Therefore, we tested the cell lines for their capacity to bind HA (Table 3), and observed that only the CB191 cell line was able to bind HA (95%). In order to determine the direct effects of HA on these cells, we investigated variation of intracellular calcium concentration using the microfluorimetric approach.

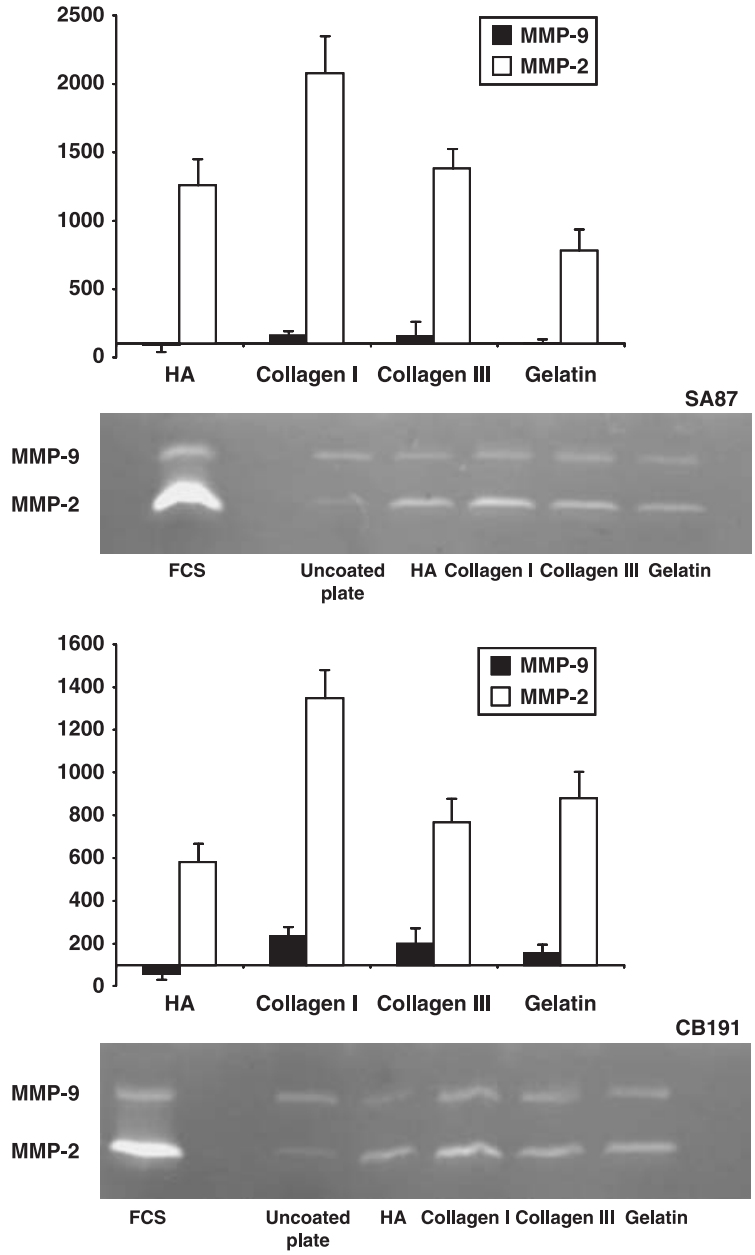

Figure 3. MMP-2 and MMP-9 activities in cancer cell culture supernatants. Gelatinolytic activities in cell-conditioned media were assessed. Foetal calf serum, shown to contain MMP-2 and MMP-9, used as positive control. Zymograms are representative results chosen from three independent experiments. Inserts: mean intensity (±SEM) of zymographic bands representing MMP-2 and MMP-9 secretion in the presence of pure HA hydrogel or coated with type I collagen, type III collagen or gelatin. Results are normalized to control band intensity (uncoated culture dishes) considered as 100%.

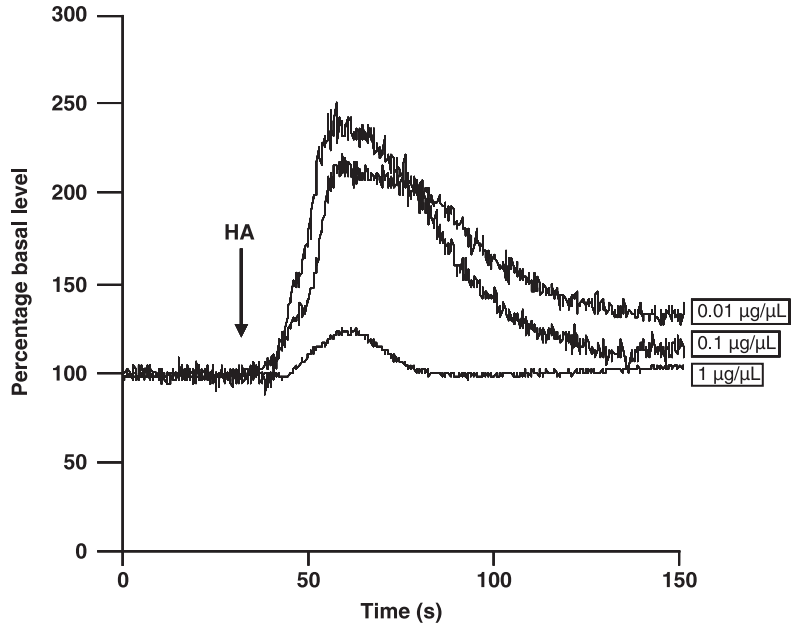

Figure 4. Effect of soluble HA on cytosolic calcium mobilization. Intracellular calcium concentration $[Ca^{2+}]_i$ in SA87 cancer cells monitored by microfluorimeter system. HA was injected in the vicinity of cells by a pressure-ejected system. Curves represent mean of results obtained with 10 SA87 cells.

Despite lack of capacity to bind HA, all unlabelled cells responded to HA by an increase in $[Ca^{2+}]_i$ and, surprisingly, the HA-binding CB191 cell line was unable to respond to presence of the same stimuli. Figure 4 shows three dose-dependent kinetic plots of HA-induced calcium mobilization. These profiles represent means of several individual responses observed in non-HA-binding SA87 cells. Extracellular application of HA (0.01 µg/µL) provoked a pronounced, transient increase in $[Ca^{2+}]_i$, reaching 2226 nM before recovering the basal level within 100 s. With concentrations of 0.1 and 1 µg/µL, HA evoked weaker and shorter responses. For other HA concentrations (lower than 0.01 µg/µL or higher than 1 µg/µL) or HA fragments used as control, we did not observe the response. In addition, responses were instantaneously observed after the HA injection (< 5 s).

Effect of TAPI on invasiveness

Because HA can trigger a Ca^{2+} response in SA87, SA87M1 and SA87M2 cells, these lines must present HA-binding sites. Thus, absence of staining with the HA-binding site assay could be due to shedding of CD44 after binding of HA; ADAM-10 and ADAM-17 are known to be involved in the shedding of CD44 (Nakamura *et al*. 2004). In order to confirm the role of HA-binding capacity and CD44 shedding, we tested the effect of TAPI (an ADAM inhibitor) on cancer cell invasiveness. SA87, SA87M1 and SA87M2 cells exhibited a significant decrease in invasiveness after TAPI treatment (59.84%, $P = 0.0002$; 35.88%, $P = 0.05$; and 31.13%, $P = 0.05$, respectively; Fig. 5). In contrast, in the HA-binding cell line CB191, no significant change in invasiveness after TAPI treatment occurred. Moreover, as shown in Table 3, the cells that were unlabelled in HA-binding tests, appeared to be labelled in the presence of TAPI.

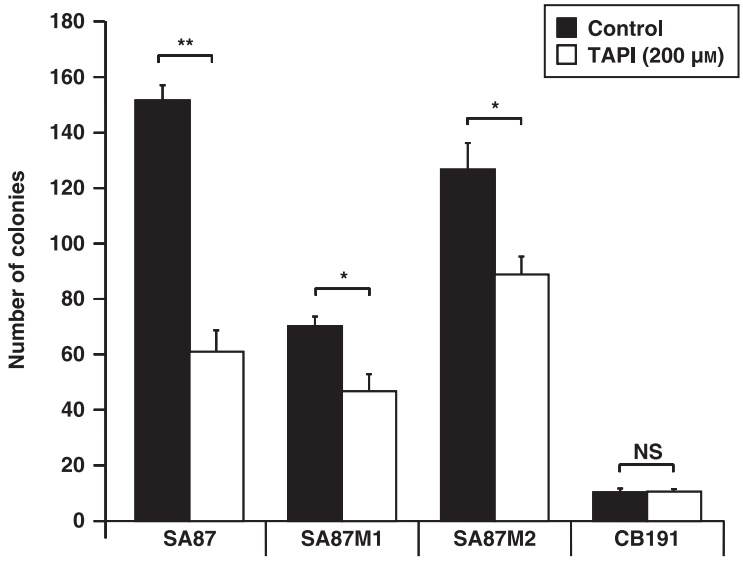

Figure 5. TAPI effect on invasiveness in HA hydrogels. TAPI was added to the culture medium (200 μM). The number of colonies of more than 150 μm was determined for each hydrogel at day 5 with the use of an inverted microscope. Results are represented as number of colonies per hydrogel as mean ± SEM of values obtained from five independent experiments (*$P < 0.05$; **$P < 0.01$).

Chemotactic and chemokinetic activities of SDF-1α and bFGF on cancer cell lines

Chemotaxis and chemokinesis are, respectively, defined as directional cell movement towards concentration gradients of solubilized attractants (SDF-1α and bFGF) and as a random cell movement in the absence of gradients (Liu & Klominek 2004).

In this set of experiments, we investigated whether SDF-1α and bFGF possessed both characteristics in stimulation of cellular invasiveness. It was observed (Fig. 6) that in the positive gradient configuration (when factors were present only in reticulated space of hydrogel) both cytokines were able to significantly stimulate hydrogel colonization (SA87 $P < 0.0009$ with SDF-1α and $P < 0.0006$ with bFGF; CB191 $P < 0.0002$ with SDF-1α and $P < 0.0001$ with bFGF). In contrast, when each factor was present in both compartments (hydrogel and medium) only bFGF could stimulate cellular expansion (SA87 $P < 0.0005$; CB191 $P < 0.0005$).

DISCUSSION

There is increasing evidence that tumour growth is the result of cooperation between transformed cells and all ECM elements conditioning many cellular events, such as differentiation, proliferation, apoptosis or angiogenesis (Condon 2005). If classical bidimensional cultures have not been very appropriate at reproducing complexity of cell/ECM interactions, the recent introduction of 3D polymeric scaffolds seems to offer a valuable solution to study such relationships (David *et al*. 2004; Doillon *et al*. 2004; Grayson *et al*. 2004). Such a three-dimensional matrix allows *ex vivo* formation of continuous histotypic organization imitating *in vivo* tissue organization. Several studies have shown the efficiency of three-dimensional models in growth of

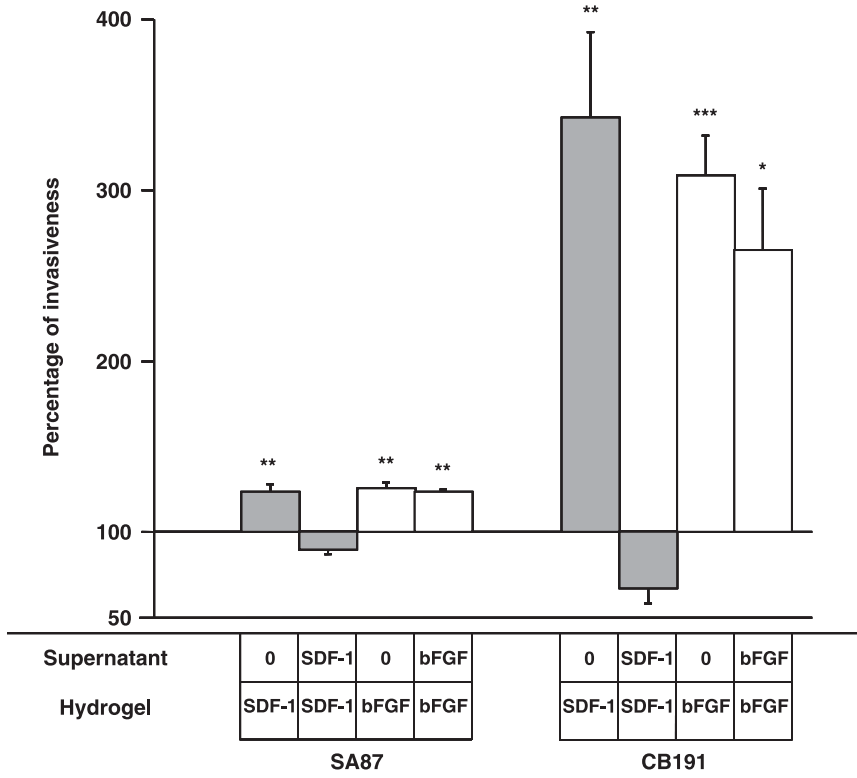

Figure 6. Effect of bFGF and SDF-1α on cell invasiveness in HA hydrogels. SDF-1α (100 ng/mL) or bFGF (25 ng/mL) were added to the hydrogel (positive gradient in order to study chemotaxis) or both hydrogel and supernatant (absence of gradient in order to study chemokinesis). Number of colonies of more than 150 μm was determined for each hydrogel at day 5 using an inverted microscope. Results normalized as number of colonies in hydrogel in presence of SDF-1α or bFGF to number of colonies in single hydrogel (for SA87, 100% correspond to 159 colonies and, for CB191, to 13 colonies). Results are mean ± SEM of values obtained from five independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$).

vascular smooth muscle cells or in vessel formation by endothelial cells (Ramamurthi & Vesely 2002; Tonello *et al*. 2003).

Hyaluronan and collagens appear to be central components of stroma that surround and supports tumour growth (Cukierman *et al*. 2002; Toole 2004). In an attempt to understand its role, we have developed our three-dimensional scaffold based on HA and different types of collagen, in which the degree of reticulation was optimized for cell migration and proliferation (data not shown). We used these hydrogels to clarify a number of issues involving HA, collagens, bFGF and SDF-1α in the migration and proliferation of cancer cells.

Our results provide strong evidence for a direct role of striated fibrillar collagens (types I and III collagens) in tumour growth. Cells were more invasive in the presence of type III collagen than types IV and V, which represent the structural backbone of basement membranes of several solid organs. Our results are in agreement with *in vivo* observations, indicating that stroma produced in the vicinity of a neoplastic process, at sites of active tumour invasion, is rich in type III collagen but not in type I collagen and fibronectin (Sivridis *et al*. 2004). Invasion could also depend on the number of cancer cells that attach to uncoated or coated hydrogels. However, we have seen that there was no significant difference in adhesion between the four tested collagens;

therefore, results observed were not due to initial cell adherence but to signalling exerted during growth. As these proteins (types I and III collagens) have the ability to modify different cellular activities (Jussila *et al*. 2004), we studied their implication in the release of MMPs that are recognized to facilitate metastatic tumour progression (Foda & Zucker 2001). Zymographic analysis showed that HA may act as an autonomous stimulatory factor of MMP-2 secretion and was interesting to note that type I collagen may also be considered as a relatively potent stimulator of MMP-2 release in our three-dimensional matrix. This original finding was recently reported by Fichter *et al*. who showed that the N-terminal fragment of type II collagen, tested on cultured articular chondrocytes, was able to stimulate the production of both mRNA and proteins corresponding to several metalloproteinases, including MMP-2, -3, -9 and -13 (Fichter *et al*. 2006). Because MMP-2 and MMP-9 are known to be implicated in proteolysis of types IV and V collagens (Nabeshima *et al*. 2002) that constitutes blood vessel basement membrane, our results suggest that HA and collagen may play an active role in tumour cell intra- and extravasation determining the metastatic process. On the contrary, increase of invasiveness induced by gelatin on the CB191 cell line could involve other signalling events, because the RGD peptides (arginine-glycine-aspartic acid) of gelatin are exposed to integrins without activation of MMP (Kojima *et al*. 2003).

Altogether, these findings indicated that invasive cells respond to soluble HA by Ca^{2+}-triggered secretion of ADAM. By cleaving the CD44 protein, ADAM decreases cellular adhesion to HA-rich matrix and favours the hydrogel colonization. The implication of ADAM-10 and ADAM-17 activities in CD44 hydrolysis was recently described in human glioma cells (Nagano *et al*. 2004), in which both enzymes were found to be, respectively, controlled by a Ca^{2+}-dependent calmodulin kinase and protein kinase C. It was also shown that Ca^{2+} influx-induced cleavage of CD44 is required for cellular migration in the ECM space. In cell models used in the present work, understanding the transductional mechanism of CD44 receptors is now in progress.

Because both ADAM-10 and ADAM-17 have been implicated in tumour cell invasion (Noel *et al*. 2004), the current study could provide an interesting link among these ADAMs, CD44, HA and tumour cell invasion. Presumably, lack of cleavage of CD44 receptors expressed in the CB191 cell line, together with the absence of response to HA, suggested the involvement of an a further mechanism of transduction that would impose a non-migratory cell state, in essence 'locking' them. As these cells became invasive in the presence of collagens, it is conceivable to think that there exists an intracellular loop between HA and collagen-inducing pathways.

Chemokines acting through chemotactic and chemokinetic mechanisms may have a crucial role in tumour progression and localization (Ferris & Wang 2003; Balkwill 2004; Kim *et al*. 2004). Histological reports have shown that in epithelial tumours, the matrix is significantly enriched in chemokines (Balkwill 2004). In our experimental model, we observed that bFGF had both chemotactic and chemokinetic activities, while SDF-1α had only a chemotactic effect. The metastatic process involves a coordinated series of interactions between tumour cells and the surrounding ECM; chemokines and growth factors embedded in the matrix determine the fate of normal cells. Alterations in binding of a cell to the ECM could enable tumour cells to proliferate, migrate, invade the matrix and colonize distant sites.

The HA hydrogel, therefore, provides a valid *in vitro* model to study tumour cell behaviour that reproduces salient features of the natural tumour microenvironment. Action of collagens and chemokines were shown to be critical for invasion in the HA hydrogel system and, therefore, specific questions pertaining to the behaviour of larger molecules in a three-dimensional setting with particular reference to the possible role of cell–cell and cell–ECM interactions, their penetration and therapeutic effects exist. Experiments in progress using the HA hydrogel coated with other ECM components will allow better understanding of the mechanisms involved in tumour initiation and invasion.

In conclusion, the results presented in this study indicate that collagen and HA may equally contribute to invasiveness capacity of tumour cells. Both compounds possess the ability to induce secretion of MMP-2 that is recognized as a factor for poor cancer prognosis. The mechanism of ADAM-induced CD44 hydrolysis seems to be essential in the initiation of cellular invasiveness.

ACKNOWLEDGEMENTS

L. David is a recipient of a fellowship from the Conseil Régional de Haute Normandie. This study was supported by grants from la région Haute-Normandie, le réseau MIIAT 'Matrice Extracellulaire Normale et Pathologique' and l'Association 'Vie et Espoir'.

REFERENCES

Assmann V, Gillett CE, Poulsom R, Ryder K, Hart IR, Hanby AM (2001) The pattern of expression of the microtubule-binding protein RHAMM/IHABP in mammary carcinoma suggests a role in the invasive behaviour of tumour cells. *J. Pathol*. **195**, 191–196.

Auvinen P, Tammi R, Parkkinen J, Tammi M, Agren U, Johansson R, Hirvikoski P, Eskelinen M, Kosma VM (2000) Hyaluronan in peritumoural stroma and malignant cells associates with breast cancer spreading and predicts survival. *Am. J. Pathol*. **156**, 529–536.

Balkwill F (2004) The significance of cancer cell expression of the chemokine receptor CXCR4. *Semin. Cancer Biol*. **14**, 171–179.

Chandler LA, Sosnowski BA, Greenlees L, Aukerman SL, Baird A, Pierce GF (1999) Prevalent expression of fibroblast growth factor (FGF) receptors and FGF2 in human tumor cell lines. *Int. J. Cancer* **81**, 451–458.

Condon MS (2005) The role of the stromal microenvironment in prostate cancer. *Semin. Cancer Biol*. **15**, 132–137.

Cukierman E, Pankov R., Yamada KM (2002) Cell interactions with three-dimensional matrices. *Curr. Opin. Cell Biol*. **14**, 633–639.

David L, Dulong V, Le Cerf D, Chauzy C, Norris V, Delpech B, Lamaczi M, Vannier JP (2004) Reticulated hyaluronan hydrogels: a model for examining cancer cell invasion in 3D. *Matrix Biol*. **23**, 183–193.

Delpech B, Bertrand P, Maingonnat C, Girard N, Chauzy C (1995) Enzyme-linked hyaluronectin: a unique reagent for hyaluronan assay and tissue location and for hyaluronidase activity detection. *Anal. Biochem*. **229**, 35–41.

Delpech B, Girard N, Bertrand P, Courel MN, Chauzy C, Delpech A (1997a) Hyaluronan: fundamental principles and applications in cancer. *J. Intern. Med*. **242**, 41–48.

Delpech B, Girard N, Olivier A, Maingonnat C, van Driessche G, van Beeumen J, Bertrand P, Duval C, Delpech A, Bourguignon J (1997b) The origin of hyaluronectin in human tumors. *Int. J. Cancer* **72**, 942–948.

Doillon CJ, Gagnon E, Paradis R, Koutsilieris M (2004) Three-dimensional culture system as a model for studying cancer cell invasion capacity and anticancer drug sensitivity. *Anticancer Res*. **24**, 2169–2177.

Eisenhardt A, Frey U, Tack M, Rosskopf D, Lummen G, Rubben H, Siffert W (2005) Expression analysis and potential functional role of the CXCR4 chemokine receptor in bladder cancer. *Eur. Urol*. **47**, 111–117.

Ferris R, Wang J (2003) Chemokine expression and roles in head and neck cancer. *Arch. Otolaryngol. Head Neck Surg*. **129**, P160.

Fichter M, Korner U, Schomburg J, Jennings L, Cole AA, Mollenhauer J (2006) Collagen degradation products modulate matrix metalloproteinase expression in cultured articular chondrocytes. *J. Orthop. Res*. **24**, 63–70.

Foda HD, Zucker S (2001) Matrix metalloproteinases in cancer invasion, metastasis and angiogenesis. *Drug Discov. Today* **6**, 478–482.

Galas L, Garnier M, Lamacz M (2000) Calcium waves in frog melanotrophs are generated by intracellular inactivation of TTX-sensitive membrane Na+ channel. *Mol. Cell. Endocrinol*. **170**, 197–209.

Gontero P, Banisadr S, Frea B, Brausi M (2004) Metastasis markers in bladder cancer: a review of the literature and clinical considerations. *Eur. Urol*. **46**, 296–311.

Grayson WL, Ma T, Bunnell B (2004) Human mesenchymal stem cells tissue development in 3D PET matrices. *Biotechnol. Prog*. **20**, 905–912.

Jackson AP, Timmerman MP, Bagshaw CR, Ashley CC (1987) The kinetics of calcium binding to fura-2 and indo-1. *FEBS Lett*. **216**, 35–39.

Janowska-Wieczorek A, Marquez LA, Nabholtz JM, Cabuhat ML, Montano J, Chang H, Rozmus J, Russell JA, Edwards DR, Turner AR (1999) Growth factors and cytokines upregulate gelatinase expression in bone marrow CD34(+) cells and their transmigration through reconstituted basement membrane. *Blood* **93**, 3379–3390.

Jussila T, Kauppila S, Bode M, Tapanainen J, Risteli J, Risteli L, Kauppila A, Stenbäck F (2004) Synthesis and maturation of type I and type III collagens in endometrial adenocarcinoma. *Eur. J. Obstet. Gynecol. Reprod. Biol*. **115**, 66–74.

Kim J, Mori T, Lam S, Nguyen L, Morton D, Bilchik A, Hoon D (2004) CXCR4 chemokine receptor mediates melanoma and colorectal cancer metastasis to the liver. *J. Am. Coll. Surg*. **199**, 81.

Kim HR, Wheeler MA, Wilson CM, Iida J, Eng D, Simpson MA, McCarthy JB, Bullard KM (2004) Hyaluronan facilitates invasion of colon carcinoma cells *in vitro* via interaction with CD44. *Cancer Res*. **64**, 4569–4576.

Kincade PW, Zheng Z, Katoh S, Hanson L (1997) The importance of cellular environment to function of the CD44 matrix receptor. *Curr. Opin. Cell Biol*. **9**, 635–642.

Klein G, Vellenga E, Fraaije MW, Kamps WA, de Bont ESJM (2004) The possible role of matrix metalloproteinase (MMP)-2 and MMP-9 in cancer, e.g. acute leukemia. *Crit. Rev. Oncol. Hematol*. **50**, 87–100.

Knudson W, Chow G, Knudson CB (2002) CD44-mediated uptake and degradation of hyaluronan. *Matrix Biol*. **21**, 15–23.

Kojima T, Koide T, Nagata H, Inamura Y, Sano M, Ito N, Suzumura K, Hasegawa M (2003) Effect of gelatins on human cancer cells *in vitro*. *Cancer Biother. Radiopharm*. **18**, 147–155.

Kucia M, Jankowski K, Reca R, Wysoczynski M, Bandura L, Allendorf DJ, Zhang J, Ratajczak J, Ratajczak MZ (2004) CXCR4-SDF-1 signalling, locomotion, chemotaxis and adhesion. *J. Mol. Histol*. **35**, 233–245.

Lakka SS, Gondi CS, Yanamandra N, Olivero WC, Dinh DH, Gujrati M, Rao JS (2004) Inhibition of cathepsin B and MMP-9 gene expression in glioblastoma cell line via RNA interference reduces tumor cell invasion, tumor growth and angiogenesis. *Oncogene* **23**, 4681–4689.

Liu Z, Klominek J (2004) Chemotaxis and chemokinesis of malignant mesothelioma cells to multiple growth factors. *Anticancer Res*. **24**, 1625–1630.

Maaser K, Wolf K, Klein CE, Niggemann B, Zanker KS, Brocker EB, Friedl P (1999) Functional hierarchy of simultaneously expressed adhesion receptors: integrin alpha2beta1 but not CD44 mediates MV3 melanoma cell migration and matrix reorganization within three-dimensional hyaluronan-containing collagen matrices. *Mol. Biol. Cell* **10**, 3067–3079.

Marhaba R, Zoller M (2004) CD44 in cancer progression: adhesion, migration and growth regulation. *J. Mol. Histol*. **35**, 211–231.

Maunoury R (1977) Establishment and characterization of 3 human cell lines derived from intracerebral metastatic tumors. *C. R. Acad. Sci. Hebd. Seances Acad. Sci. D* **284**, 991–994.

Nabeshima K, Inoue T, Shimao Y, Sameshima T (2002) Matrix metalloproteinases in tumor invasion: role for cell migration. *Pathol. Int*. **52**, 255–264.

Nagano O, Saya H (2004) Mechanism and biological significance of CD44 cleavage. *Cancer Sci*. **95**, 930–935.

Nagano O, Murakami D, Hartmann D, De Strooper B, Saftig P, Iwatsubo T, Nakajima M, Shinohara M, Saya H (2004) Cell-matrix interaction via CD44 is independently regulated by different metalloproteinases activated in response to extracellular Ca(2+) influx and PKC activation. *J. Cell Biol*. **165**, 893–902.

Nakamura H, Suenaga N, Taniwaki K, Matsuki H, Yonezawa K, Fujii M, Okada Y, Seiki M (2004) Constitutive and induced CD44 shedding by ADAM-like proteases and membrane-type 1 matrix metalloproteinase. *Cancer Res*. **64**, 876–882.

Naor D, Nedvetzki S, Golan I, Melnik L, Faitelson Y (2002) CD44 in cancer. *Crit. Rev. Clin. Lab. Sci*. **39**, 527–579.

Noel A, Maillard C, Rocks N, Jost M, Chabottaux V, Sounni NE, Maquoi E, Cataldo D, Foidart JM (2004) Membrane associated proteases and their inhibitors in tumour angiogenesis. *J. Clin. Pathol*. **57**, 577–584.

Ponta H, Sherman L, Herrlich PA (2003) CD44: from adhesion molecules to signalling regulators. *Nat. Rev. Mol. Cell Biol*. **4**, 33–45.

Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M (2005) Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev*. **16**, 159–178.

Prestwich GD, Marecak DM, Marecek JF, Vercruysse KP, Ziebell MR (1998) Controlled chemical modification of hyaluronic acid: synthesis, applications, and biodegradation of hydrazide derivatives. *J. Control. Release* **53**, 93–103.

Ramamurthi A, Vesely I (2002) Smooth muscle cell adhesion on crosslinked hyaluronan gels. *J. Biomed. Mater. Res*. **60**, 195–205.

Ropponen K, Tammi M, Parkkinen J, Eskelinen M, Tammi R, Lipponen P, Ågren U, Alhava E, Kosma VM (1998) Tumor cell-associated hyaluronan as an unfavorable prognostic factor in colorectal cancer. *Cancer Res*. **58**, 342–347.

Sivridis E, Giatromanolaki A, Koukourakis MI (2004) 'Stromatogenesis' and tumor progression. *Int. J. Surg. Pathol*. **12**, 1–9.

Stern R (2005) Hyaluronan metabolism: a major paradox in cancer biology. *Pathol. Biol*. **53**, 372–382.
Su L, Zhang J, Xu H, Wang Y, Chu Y, Liu R, Xiong S (2005) Differential expression of CXCR4 is associated with the metastatic potential of human non-small cell lung cancer cells. *Clin. Cancer Res*. **11**, 8273–8280.
Tonello C, Zavan B, Cortivo R, Brun P, Panfilo S, Abatangelo G (2003) *In vitro* reconstruction of human dermal equivalent enriched with endothelial cells. *Biomaterials* **24**, 1205–1211.
Toole BP (2004) Hyaluronan: from extracellular glue to pericellular cue. *Nat. Rev. Cancer* **4**, 528–539.
Toole BP, Wight TN, Tammi MI (2002) Hyaluronan-cell interactions in cancer and vascular disease. *J. Biol. Chem*. **277**, 4593–4596.
Tun T, Miyoshi H, Aung T, Takahashi S, Shimizu R, Kuroha T, Yamamoto M, Ohshima N (2002) Effect of growth factors on *ex vivo* bone marrow cell expansion using three-dimensional matrix support. *Artif. Organs* **26**, 333–339.
Turpeenniemi-Hujanen T (2005) Gelatinases (MMP-2 and -9) and their natural inhibitors as prognostic indicators in solid cancers. *Biochimie* **87**, 287–297.
Vizoso FJ, del Casar JM, Corte MD, Garcia I, Corte MG, Alvarez A, Garcia-Muniz JL (2004) Significance of cytosolic hyaluronan levels in gastric cancer. *Eur. J. Surg. Oncol*. **30**, 318–324.

